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# ***Mangifera indica* L. Leaves Extract Induced Intrinsic Apoptotic Pathway in MCF-7 Cells by Decreasing Bcl-2 Expression and Inducing Bax Expression**

Fitriasih<sup>1</sup>, Siti Mega Komariyah<sup>1</sup>, Ferry Sandra<sup>2</sup>, Nenny Pratiwi<sup>1</sup>, Devi Nisa Hidayati<sup>1,\*</sup>

<sup>1</sup>Faculty of Pharmacy, Universitas Wahid Hasyim, Semarang, Indonesia

<sup>2</sup>Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia

## **Abstract**

Extract of *Mangifera indica* L. has been investigated to have cytotoxic activity against MCF-7 cells. Current research was conducted to determine the cytotoxic activity of methanol extract of *M. indica* leaves (MEML) on MCF-7 breast cancer cells and to investigate on the MEML's effect on Bcl-2 and Bax expressions. *M. indica* leaves were extracted using soxhletation with methanol. The cytotoxic test was performed using MTT assay with various MEML concentrations: 15.625, 31.25, 62.5, 125, 250 and 1000 µg/mL. Meanwhile, Bax and Bcl-2 expressions of MCF-7 breast cancer cells were detected with immunocytochemical staining after treatment of MEML with concentrations of  $\frac{1}{4}$  IC<sub>50</sub>,  $\frac{1}{2}$  IC<sub>50</sub> and IC<sub>50</sub>. The results showed that IC<sub>50</sub> of cytotoxic activity was 308.12 µg/mL. MEML could influence Bax and Bcl-2 protein expressions. After treatment of MEML, Bcl-2 expression was decreased while Bax expression was increased.

**Keywords :** *Mangifera indica* L., Cytotoxic, Bax, Bcl-2, MCF-7

## **INTRODUCTION**

Based on Global Cancer Observatory Fact sheets, breast cancers is ranked 1 among all other cancers in Indonesia. It has been calculated that the breast cancer age-standardized rate (ASR) per 100,000 is 42.1 and the number of new cases in 2018 is 58,256 cases (16.7%) (Globocan, 2018). Multi-agent chemotherapy improved overall survival rate, however, the use of chemotherapy might cause side effects (Sandra and Sidharta, 2018). Therefore exploration to find the chemotherapeutic agents that have high cytotoxic activity and low side effects is necessary.

Mango is one of the plants that has anti-cancer activity. Abdullah, *et al.*, (2014) proved ethanol

kernel extract of *Mangifera indica* L. had cytotoxic activity against MCF-7 and MDA-MB-231 cells. Barreto, *et al.*, (2008) identified polyphenol compound namely mangiferin, which is highly contained in leaves. Núñez Selles, *et al.* (2014) stated that mangiferin inhibits the activation of the nuclear factor (NF)-κB, which has a crucial role in oxidative processes, chronic inflammation and anti-apoptosis (Hendarmin, *et al.*, 2008).

The mechanism of apoptosis occurs through two pathways, through mitochondria known

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\*Corresponding author: devinisahidayati@yahoo.com

as intrinsic pathway (Sandra, 2014) and death receptors on the cell surface, known as extrinsic pathway (Sandra, 2018). Changes in the potential of mitochondrial membranes depend on the ratio between proapoptotic protein (Bax) and anti-apoptotic protein (Bcl-2) (Sandra, et al., 2001), which will decide the cell fate. MCF-7 breast cancer cell line has been reported to have Bcl-2 and Bax overexpressions (Adefolaju, et al., 2010). Based on these studies, we conducted a study to investigate the cytotoxic activity and expressions of Bax and Bcl-2 of MCF-7 cells treated with *M. indica* L. leaves extract.

## MATERIALS AND METHODS

### Extract Preparation and Cell Culture

Fresh and healthy leaves of *M. indica* L. were harvested from Wilalung village RT 2 RW 6 Gajah district Demak regency. The leaves were dried and further processed with soxhletation method using methanol to obtain methanol extract of *M. indica* leaves (MEML).

MCF-7 breast cancer cells were obtained from the Laboratory of Parasitology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin (Gibco).

### 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl-tetrazolium Bromide (MTT) Assay

In each well of 96-well plate, 5,000 MCF-7 cells were seeded and incubated overnight. Then, MEML was added to reach the concentrations of 15.625, 31.25, 62.5, 125, 250, 1000 µg/mL for 24 hours. After the incubation, 0.5 mg/mL MTT reagent was added into each well, then incubated at 37°C for approximately 3 hours. Then added 10% SDS in 0.1 N HCl was added and incubated overnight in dark. The absorbance was measured by using an ELISA reader at λ 595 nm.

## Immunocytochemistry

MCF-7 cells were seeded in 6-well plates with  $5 \times 10^4$  cells/well density on a coverslip. Upon reaching 80% confluent, the cells were treated with  $\frac{1}{4}$  IC<sub>50</sub>,  $\frac{1}{2}$  IC<sub>50</sub> and IC<sub>50</sub> of MEML for 24 hours. Then, the cells were washed, fixed with methanol, and treated with 3.7% H<sub>2</sub>O<sub>2</sub> in methanol for 10 minutes.

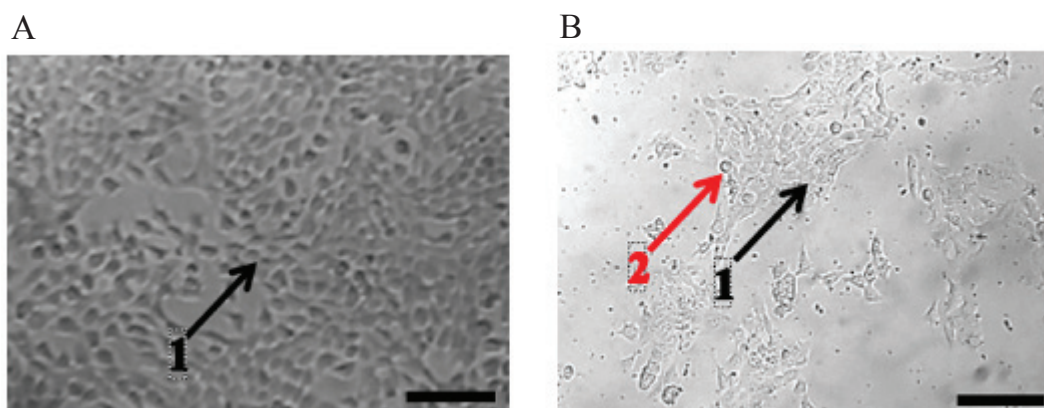
Afterthat, the cells were washed and treated with blocking serum for 15 minutes. The cells were further processes with mouse monoclonal anti-Bcl-2 (Dako, Carpinteria, CA, USA) or rabbit polyclonal anti-Bax (Dako) antibody for 60 minutes, biotinylated universal secondary antibody (Biocare, Pacheco, CA, USA) for 20 minutes and streptavidin HRP label (Biocare) for 10 minutes. Finally, the cells were added with DAB substrate (Dako) to visualize to the antigen-antibody bound and counterstained with hematoxylin. Expressions of Bcl-2 and Bax were observed as the brown color at the cytoplasm and membranes of MCF-7 cells under an upright light microscope. The expressions were then semiquantified using ImageJ version 1.51w software.

## RESULTS

### Morphology and Viability of MEML-treated MCF-7 Cells

The results showed that MEML had cytotoxic effect on MCF-7 cells. As shown in Figure 1, treatment of 125 µg/mL MEML for 24 hours caused morphological change of MCF-7 cells. Viable cells appeared as leaf-shape (Figure 1A), while dead cells as round (Figure 1B).

MEML treatment showed a dose-dependent phenomenon in inducing MCF-7 cell death. Percentage of viable MCF-7 cells was decreased along with the increase of MEML concentration (Figure 2). Based on the trendline equation, cytotoxic IC<sub>50</sub> of MEML on MCF-7 cells was 308.12 µg.



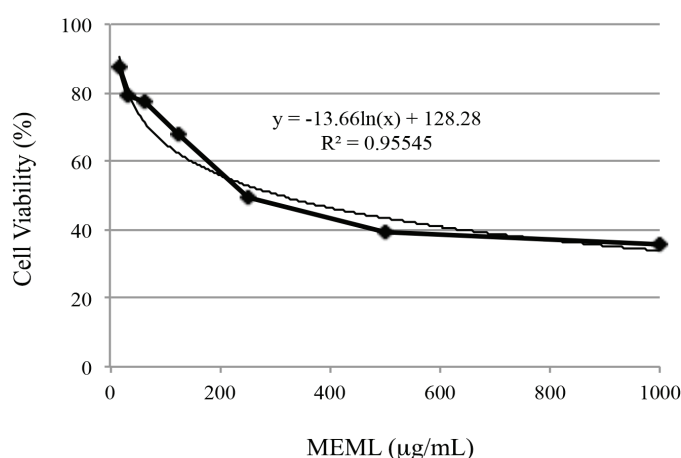
**Figure 1.** Morphology of MEML-treated MCF-7 cells. MCF-7 cells were seeded, incubated overnight and treated with 125 µg/mL MEML for 24 hours. Untreated (A) and 125 µg/mL MEML-treated (B) MCF-7 cells were documented under an inverted microscope. 1: viable cell, 2: dead cell. Bar: 100 µm.

### Bcl-2 Expressions of MEML-treated MCF-7 Cells

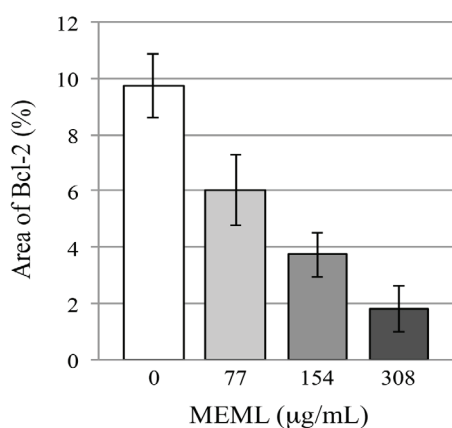
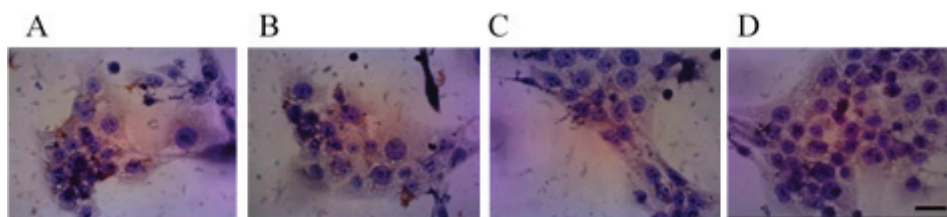
The Expression of Bcl-2 can be seen in Figures 3. The Bcl-2 Expression semiquantifications of 0, 77 ( $\frac{1}{4}$  of  $IC_{50}$ ), 154 ( $\frac{1}{2}$  of  $IC_{50}$ ) and 308 ( $IC_{50}$ ) µg/mL MEML-treated MCF-7 cells were 9.74 %, 6.03 %, 3.73 % and 1.81 %. These results showed that the treatment of 77, 154 and 308 µg/mL MEML decreased the expression of Bcl-2 until 38%, 62% and 81%, respectively.

### Bax Expressions of MEML-treated MCF-7 Cells

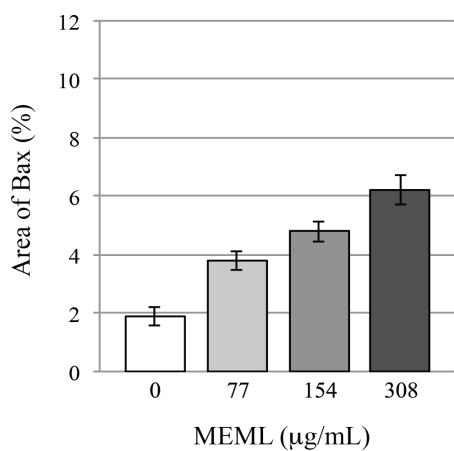
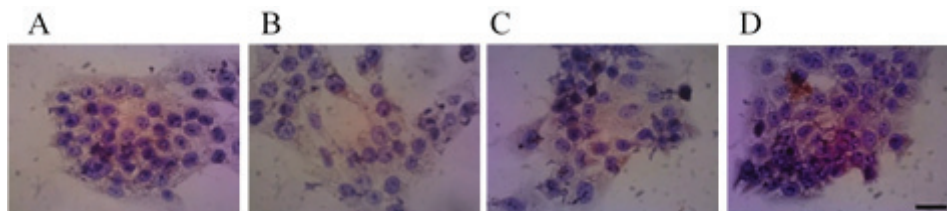
The Expression of Bax can be seen in Figures 4. The Bax Expression semiquantifications of 0, 77 ( $\frac{1}{4}$  of  $IC_{50}$ ), 154 ( $\frac{1}{2}$  of  $IC_{50}$ ) and 308 ( $IC_{50}$ ) µg/mL MEML-treated MCF-7 cells were 1.90 %, 3.78 %, 4.80 % and 6.23 %. These results showed that the treatment of 77, 154 and 308 µg/mL MEML increased the expression of Bax until 1.99, 2.53 and 3.28 folds, respectively.



**Figure 2.** Viability of MEML-treated MCF-7 cells. MCF-7 cells were seeded, incubated overnight and treated with MEML in various concentrations as shown in the figure for 24 hours. Viable cells were measured with MTT assay as described in Materials and Methods. Results were shown in line graph. Logarithmic trendline, trendline equation and R-squared value were shown in the graph as well. Each point shows the average of three replications.



**Figure 3. Bcl-2 Expression of MEML-treated MCF-7 Cells.** MCF-7 cells were treated with 0 (A), 77 (B), 154 (C) and 308 (D) µg/mL MEML. Cells were processed with Immunohistochemistry to detect Bcl-2 as described in Methods. Expressions of Bcl-2 were indicated by the presence of Bcl-2 monoclonal antibodies bonds, detected in brown color in the cytoplasm and membrane of MCF-7 cells. E: Semiquantified Bcl-2 expression with ImageJ. Bar: 50 µm.



**Figure 4. Bax Expression of MEML-treated MCF-7 Cells.** MCF-7 cells were treated with 0 (A), 77 (B), 154 (C) and 308 (D) µg/mL MEML. Cells were processed with Immunohistochemistry to detect Bax as described in Methods. Expressions of Bax were indicated by the presence of Bax monoclonal antibodies bonds, detected in brown color in the cytoplasm and membrane of MCF-7 cells. E: Semiquantified Bax expression with ImageJ. Bar: 50 µm.

## DISCUSSION

*M. Indica* L. leaves contain flavonoids, phenols, polyphenols, mangiferin, gallic acid, methyl gallate, quercetin pentoside, isoquercetin isomers (Barreto, *et al.*, 2008). Mangiferin and gallic acid both inhibited NF- $\kappa$ B activation by IKK $\alpha$ / $\beta$  (Núñez Selles, *et al.*, 2014). NF- $\kappa$ B is one of the transcription factors that regulate the expression of anti-apoptotic genes (Hendarmin, *et al.*, 2008). Regulation of intrinsic apoptosis through the regulation of Cytochrome C release. Igney and Krammer (2002) stated that the Bcl-2 expression prevents the Cytochrome C release from mitochondria. In the contrary, Bax will induce the Cytochrome C release. Within the cytosol, Cytochrome C will form a complex with Apoptotif Protease Activating Factor-1 (Apaf-1) and Procaspase-9. This complex is called apoptosome, which activates caspase-9 (Sandra, 2014). Caspase-9 activates caspase 6 and caspase-7 to execute apoptosis.

Previous research has shown that mangiferin modulates apoptosis by decreasing Bcl-2 expression and increasing Bax expression in CNE2 Nasopharyngeal Cancer Cells. Mangiferin also inhibits cell proliferation through G2/M phases (Li Li Pan, *et al.*, 2014). Current result showed that MEML induced apoptosis through the mitochondrial pathway by increasing the expression of Bax and decreasing the Bcl-2 expression, which will lead to the disruption of the mitochondrial membrane potential, then the release of Cytochrome C from the mitochondria to cytosol. However, further research is needed to confirm the suggested pathway.

## CONCLUSION

MEML induces apoptosis in MCF-7 cells through intrinsic apoptotic pathway by decreasing Bcl-2 expression and increasing Bax expression. The cytotoxic IC<sub>50</sub> of MEML on MCF-7 cells was 308.12  $\mu$ g/mL.

## ACKNOWLEDGEMENTS

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